

HISTOCHEMICAL LOCALISATION BY ELECTRON MICROSCOPY OF PECTIC SUBSTANCES IN ABCISING TISSUE

CHRIS H. BORNMAN, ARTHUR R. SPURR AND FREDRICK T. ADDICOTT

(*Department of Botany, University of Natal, Pietermaritzburg and University of California, Davis, California*)

ABSTRACT

Pectic substances may be localised in abscising plant tissue at the electron microscope level using postfixation and postsectioning rather than prefixation staining techniques. The results, though not always consistent, have considerable value in interpreting changes which occur in the pectic substances of cell walls prior to and during abscission. The non-specificity of the heavy metal dye, ruthenium red, an often used stain for pectic materials in light microscopy, appears to be borne out by electron microscopy. Alkaline hydroxylamine-ferric chloride not only is specific for pectic substances but also forms a stable, electron dense complex with the latter. The observation that auxin delays abscission by some functional regulatory relationship with the pectic substances is supported by the localisation of these substances in auxin-treated tissue of an advanced age.

INTRODUCTION

Abscission is a process in which, ultimately, breakdown of cells leads to the disjunction of plant parts. The architecture and composition of the cell wall is such that separation must involve profound chemical as well as anatomical changes, especially in the pectic components of the middle lamella. The histochemical localisation of pectic substances (Gee et al., 1959; Albersheim et al., 1960, 1963) suggested that an electron microscopic study of their distribution in the cell walls of abscising tissue might be instructive, particularly in relation to the abscission-accelerating and abscission-retarding effects of certain plant growth substances.

This paper reports on attempts to localise pectic substances with the electron microscope using a postsection rather than Albersheim's and Killias' (1963) prefixation staining technique. The obvious advantages of staining after sectioning include more uniform staining as a result of better control and a comparison of serial sections of the same tissue following different staining treatments.

Accepted for publication 12th December, 1968.

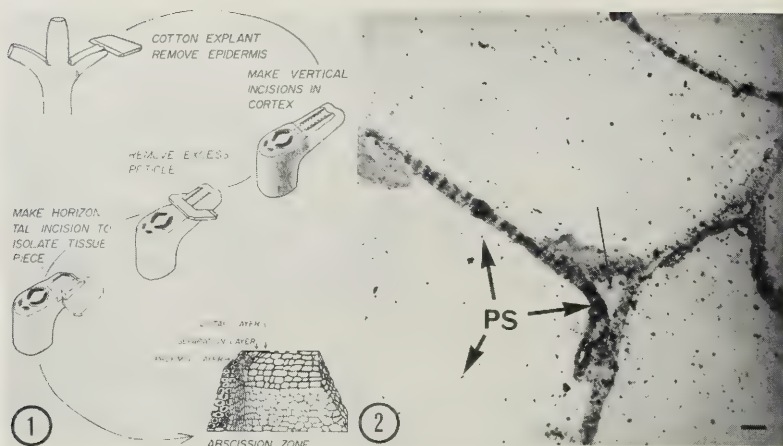


FIG. 1.
Isolation of abscission zone for electron microscopy.

FIG. 2.
Broad localisation of pectic substances (PS) in freshly excised tissue from the abscission zone of *Gossypium*.

MATERIALS AND METHODS

Tissue preparation. Tissue pieces, ca. 2 mm³, dissected from the abscission zones of variously treated *Coleus* and *Gossypium* explants (Fig. 1), were fixed for 2 hours in 3 or 6% phosphate-buffered glutaraldehyde, pH 6.8. The material was then divided into two lots, half of which was postfixed in 1 or 2% osmium tetroxide in veronal-acetate buffer, pH 6.5, for 2 hours, and half in 2% potassium permanganate for 2 hours. The osmium-fixed tissue was washed for 2 hours with veronal-acetate buffer and the permanganate-fixed material with water until the colour disappeared.

Following dehydration the tissue was infiltrated with and embedded in epoxy resin, one lot of tissue in a Maraglas preparation by a method modified from Freeman and Spurlock (1962), and the remaining lot in an Epon resin mixture (Bornman, 1967). Sections were satisfactorily cut with a diamond knife, ca. 600-800 Å thick.

Histochemical localisation of pectic substances. The localisation of pectic substances before fixation has been elegantly demonstrated at the electron microscope level by Albersheim and Killias (1963). Bornman (1967) briefly reported on the application of this technique after thin-sectioning. Upon treatment with basic hydroxylamine pectic substances form the derivative pectic

hydroxamic acids which exhibit nonselective density to electrons. However, following a reaction with a ferric salt the resulting insoluble complexes become electron dense. It appears that the amount of ferric ion is proportional to the concentration of pectic methyl ester groups rather than to total pectic substances.

Sections collected on copper and titanium grids were either floated on the staining solution, section side down, or submerged in the staining solution, section side up. The staining solution and the sequence of treatments were as follows:

Solution A: 60% solution of alkaline hydroxylamine (equal volumes of 14g NaOH per 100 ml and 14g $\text{NH}_2\text{OH}\cdot\text{HCl}$ per 100 ml) in ethanol, 2—15 minutes.

Solution B: 95% ethanol: conc. hydrochloric acid (2 : 1), gentle washing for 1—5 minutes.

Solution C: 10% ferric chloride solution in 60% ethanol containing 0.05N HCl, gentle flooding for 1—5 minutes.

Plant tissue not pretreated with basic hydroxylamine but stained directly with ferric chloride served as control.

In addition, some sections were stained by floating on or submerging in a freshly made, aqueous ruthenium red solution (1 : 10,000) for 5 hours. Ruthenium red is a dye commonly used in light microscopy to indicate the presence of pectic substances.

RESULTS AND DISCUSSION

It must be pointed out that cells in abscission zones and particularly in abscising tissue generally are highly vacuolated and frequently in an advanced state of deterioration. Good fixation and infiltration is therefore extremely difficult to achieve.

Kertesz (1951) pointed out that ruthenium red is not a stain specific for pectic substances. Furthermore, although this stain does seem to have particular affinity for highly polymerized acidic carbohydrates (Siegel, 1962) such as pectic substances, it is not known which component of the latter has an affinity for this dye.

Figure 2 depicts the walls of five adjoining cells in the distal portion of an abscission zone isolated from a freshly excised cotton seedling. A broad, non-specific pattern of localisation (PS) following staining with ruthenium red is apparent although the greater concentration of this electron dense metal is found over the cell walls. The intercellular space (unlabelled arrow) is, as may be expected, relatively free of stain and consequently of pectic substances.

Figure 3 is included to show the harsh effects on the embedment medium

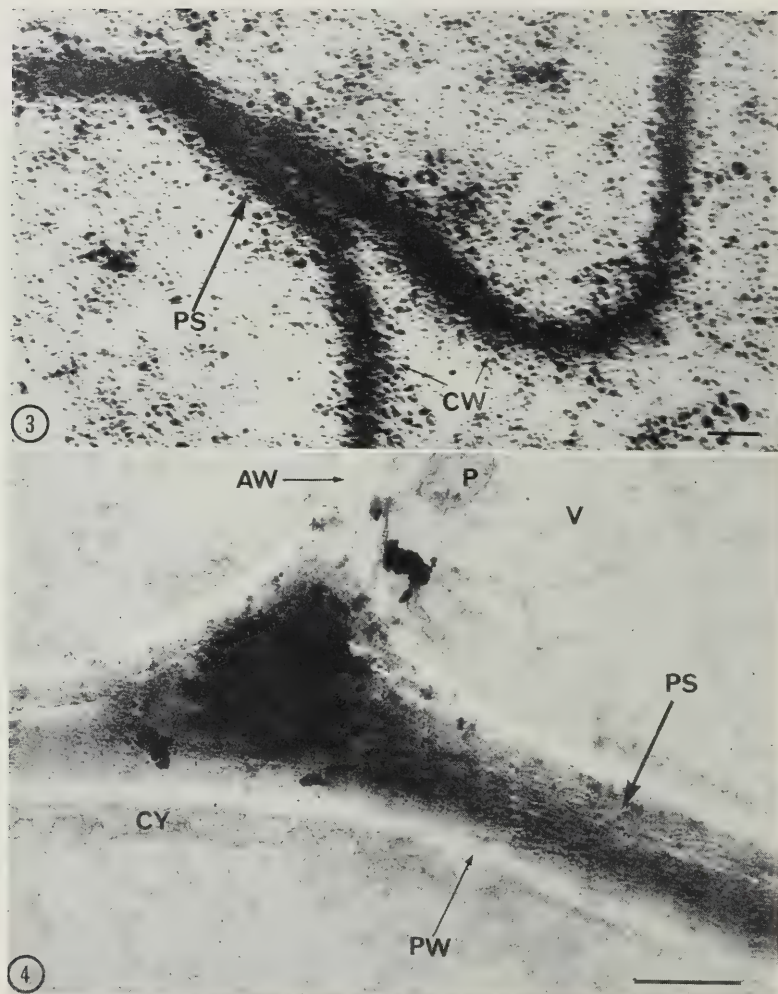
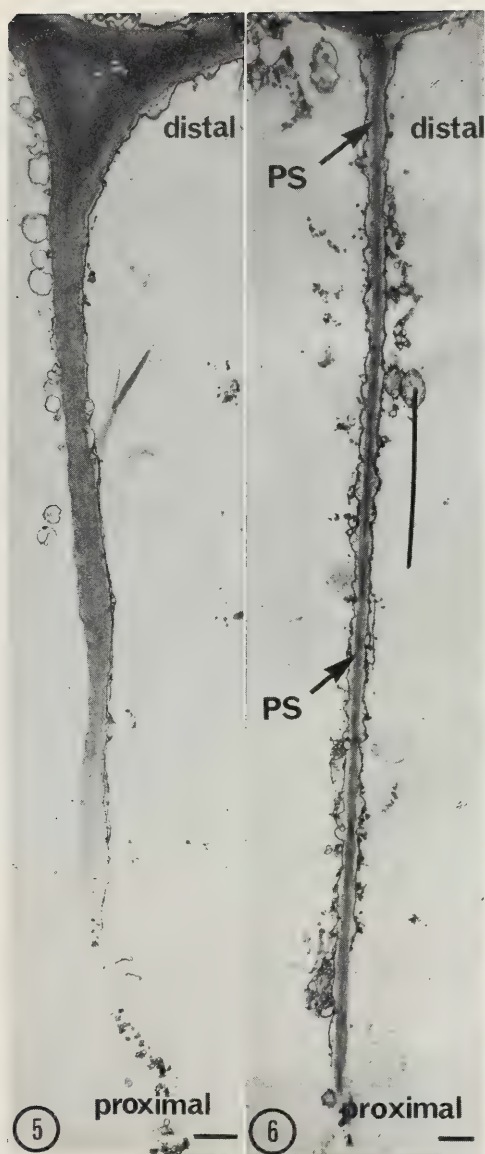


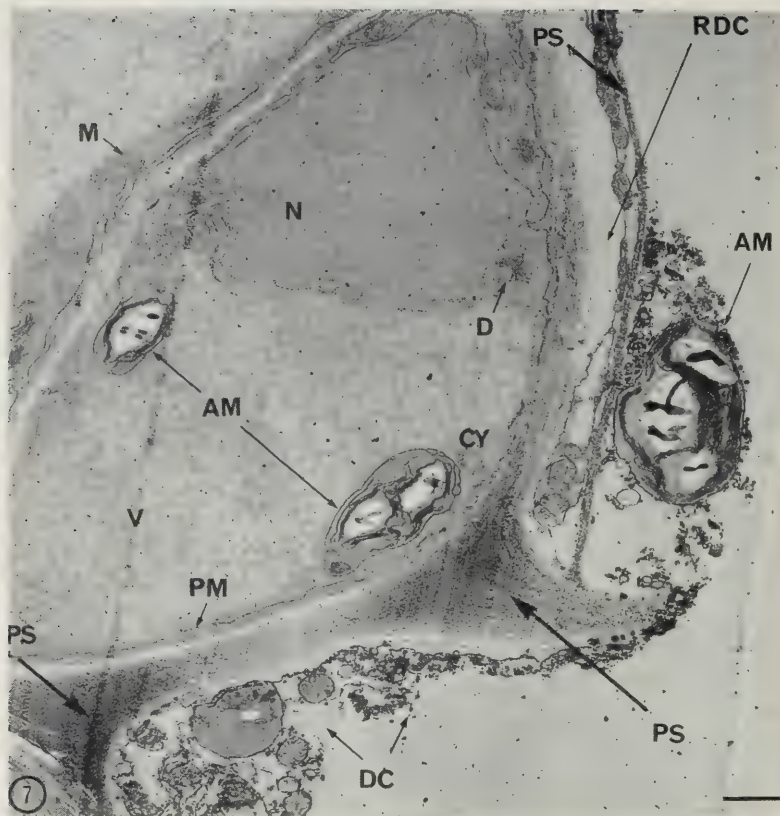
FIG. 3.
Pectic localisation (PS) in cell walls (CW) in an overstained section of freshly excised tissue from the abscission zone of *Gossypium*.

FIG. 4.
Uniform localisation of pectic substances (PS) in the middle lamella of a cell wall in a section of freshly excised tissue from the abscission zone of *Gossypium*. AW, axillary wall; CY, cytoplasm; P, plastid; PW, primary wall; V, vacuole.



FIGS. 5-6.

Cell wall breakdown in abscising tissue of *Coleus*, ca. 60-70 hours after deblading. FIG. 5: No pretreatment with alkaline hydroxylamine results in non-localisation of pectic substances. FIG. 6: Treatment with alkaline hydroxylamine followed by ferric chloride results in localisation of the remaining non-mobilised pectic substances (PS) of the middle lamella.

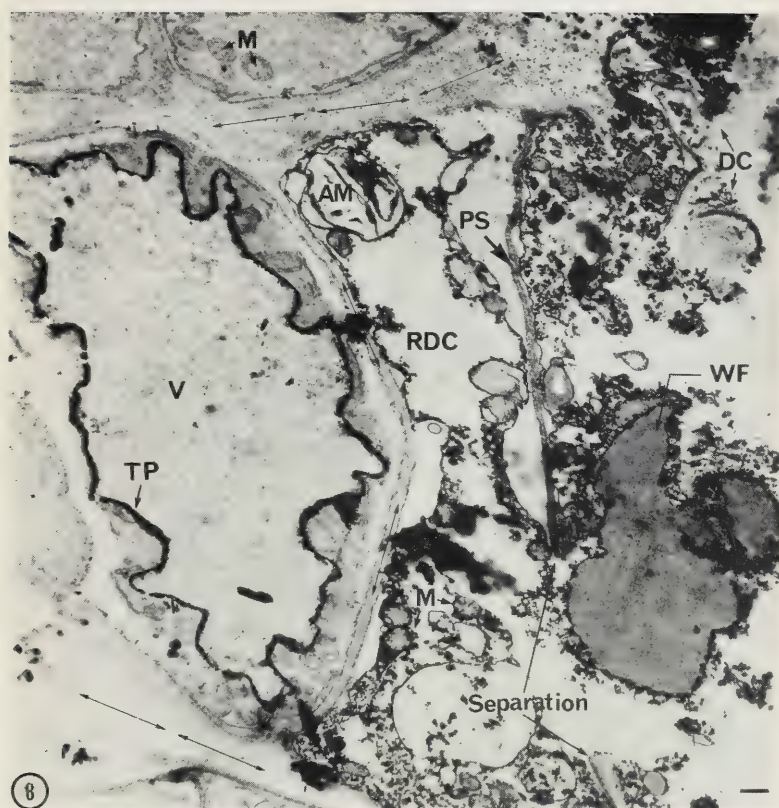


FIGS. 7-8.

Pectic substances (PS) in abscising tissue of *Gossypium* ca. 120-130 hours after treatment with indoleacetic acid at an abscission-retarding concentration. Cells are from the proximal layer. AM, amyloplast; CY, cytoplasm; D, dictyosome; DC, disintegrating cell; M, mitochondrion; N, nucleus; PM, plasma membrane; RDC, recently divided cell; WF, wall fragment.

and tissue of overstaining with ferric chloride (5 minutes). The section is from material similar to that used above (Fig. 2) but the cell contents have been destroyed by the treatment. Pectic localisation, however, is remarkably intense in a rather diffuse wall and shows at least the promise of this method.

Copper grids tended to disintegrate following treatment with FeCl_3 and proved inadequate whereas titanium grids withstood the effects of the staining chemicals.



Staining of tissue similar to that represented in Figs. 2 and 3 in Solution A for ca. 5 minutes and in Solution C for ca. 2 minutes resulted in a rather more uniform localisation of pectic substances (Fig. 4, PS), mainly in the middle lamella. The primary walls (PW), and axillary wall (AW) of a young cell, indicate a low pectic content; in the latter case probably because the plane of sectioning passed mainly through primary wall. Still, the cytoplasm (CY) and a plastid (P) appear to have been damaged during staining.

Cell walls of tissue not treated with basic hydroxylamine before staining with ferric chloride did not react positively.

Abscising tissue of *Coleus*, ca. 60—70 hours after deblading and in the cell

walls of which at this stage a considerable proportion of the pectic materials is assumed to have been mobilised, shows no staining if pretreatment with hydroxylamine is foregone (Fig. 5). However, complete alkaline hydroxylamine-ferric chloride stain localises a thin band of pectic material (PS) in the wall of an abscising cell (Fig. 6).

In both *Gossypium* and *Coleus* abscission may be retarded by application of auxin; or enhanced by conditions which lower the internal level of auxin. A functional regulatory relationship may therefore exist between auxin and the pectic substances in the abscission zone. Figure 7 shows cells in the proximal

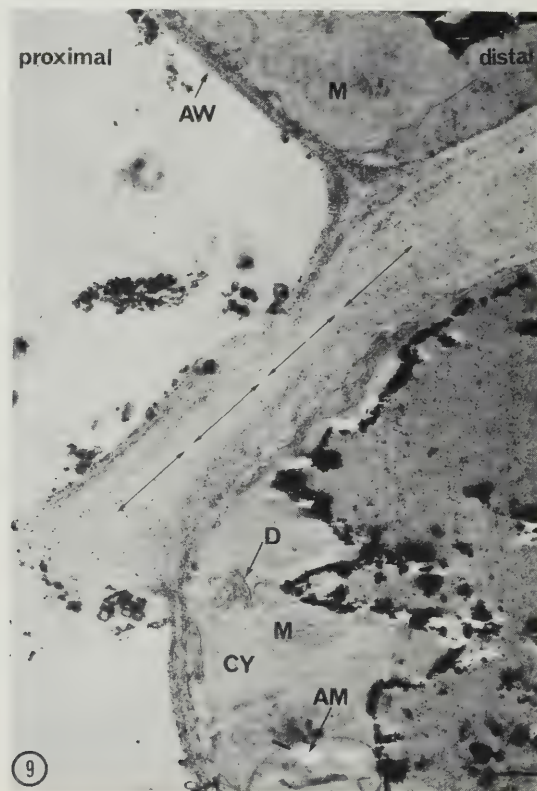
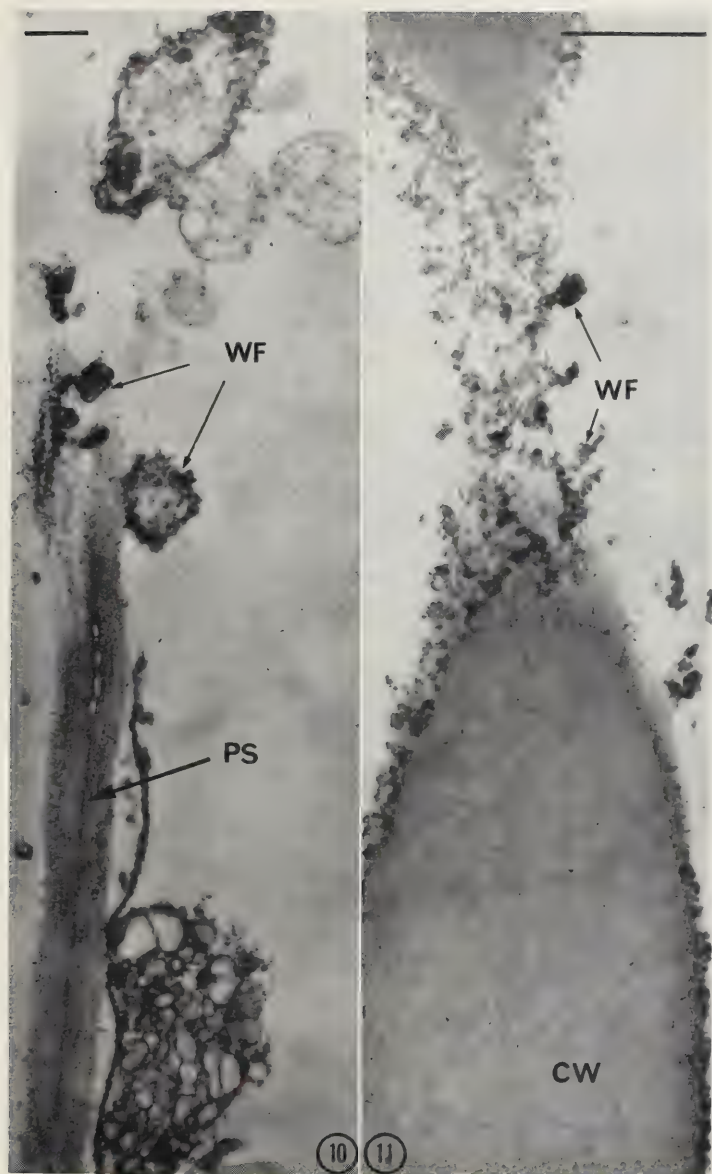


FIG. 9.

Loss of pectic substances (arrows) in abscising tissue of *Gossypium* from the distal layer ca. 120-130 hours following treatment with indoleacetic acid. AM, amyloplast; AW axillary wall; CY, cytoplasm; D, dictyosome; M, mitochondrion.



FIGS. 10-11.
Abscising tissue of *Coleus* following treatment with gibberellic acid at an abscission-accelerating concentration. Some pectic substances (PS) still present in the proximal part of the cell wall but have become mobilised in distal part. CW cell wall; WF, wall fragment.

part of an abscission zone of a cotton explant, 120–130 hours after application of 0.25 μg indoleacetic acid to the debladed petiole. Despite the flaccid state of plastids and mitochondria pectic substances are heavily localised in the wall of a recently divided cell (auxin stimulates cell division in abscission zones; Bornman et al., 1966) as well as in the wall junctions of adult cells. Although these cells are in an advanced state of deterioration, the presence of pectic material supports the concept of auxin's functional role in this regard. The cells in the separation layer in Fig. 8 are perhaps in an even more advanced stage of desiccation than those in Fig. 7. Pectic material has diminished in the adult cells. However, although the wall of the recently divided cell has been ruptured, probably as a result of mechanical stress, the contrast in intensity of pectic substances is marked especially where this wall abuts on to that of the mother cell.

On the other hand, cells on the distal side of the separation layer in the abscission zone, even in tissue treated with auxin as shown in Fig. 9, contain very little pectic material in mature cell walls (arrows); younger, transverse walls, contain somewhat more (AW). It is as a result of the partial or complete dissolution of the intercellular cementing (pectic) substance (Fig. 9) that walls of cells in the separation layer fall apart and lead to flower, fruit and leaf drop.

Figures 10 and 11 show parts of abscising cells in *Coleus* following treatment with 0.01 μg gibberellic acid per abscission zone. Some pectic substances are still present in the proximal part of the wall but have become mobilised in the distal part. Figure 11 represents a fully stained section but since it is a surface section no pectic localisation is evident. Cell wall fragments (WF) indicate the final destruction of the wall.

In general it appears that osmium-fixed tissue produces better results with this staining procedure than permanganate-fixed tissue. The oxidative action of permanganate probably is responsible for this and, in addition, further adds to the poor cytoplasmic preservation. Tissue embedded in Maraglas has given slightly better results than embedment in an Epon mixture although there is reason to assume that this is probably a function of the formulation rather than of the constituent chemicals.

This technique is somewhat erratic and inconsistent, and therefore calls for caution in the interpretation of results. However, it has considerable value as a histochemical tool.

CONCLUSIONS

The value of the histochemical approach in localising and probably quantitatively measuring substances at the subcellular level cannot be overemphasised. However, certain criteria must be imposed: the reaction should be localised as precisely as possible in relation to the structural elements of the cell and the

final product should be an insoluble chemical complex of good quality and stability which, naturally, should be capable also of scattering or deflecting electrons.

The method of staining for pectic substances following fixation and sectioning is one that appears to have merit although the technique awaits considerable refinement. Since most investigators of abscission agree that changes occur in the pectic substances preceding and during abscission, it should be possible to examine more critically the action of hormones such as abscisic acid, gibberellin and auxin on the process of abscission by investigating their effects on the pectic components of the cell wall.

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